UNCLASSIFIED

AD NUMBER

AD448488

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; AUG 1964. Other requests shall be referred to US Army Biological Labs., Attn: Technical Releases Branch/TID, Fort Detrick, Frederick, MD 21701.

AUTHORITY

US Army Biological Defense Research Lab ltr dtd 27 Sep 1971

THIS PAGE IS UNCLASSIFIED

UNCLASSIFIED

AD 448488

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA

UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

AD 448 488

TECHNICAL MANUSCRIPT 146

SUCCESSFUL TREATMENT OF RHESUS MONKEYS FOR SEPTICEMIC ANTHRAX

AUGUST 1964

DDC
RECEIVED
OCT 12 1964
DDC-IRA C

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 146

SUCCESSFUL TREATMENT OF RHESUS MONKEYS FOR SEPTICEMIC ANTHRAX

R. E. Lincoln

F. Klein

J. S. Walker

B. W. Haines

W. I. Jones, Jr.

B. G. Mahlandt

R. H. Friedman

Process Development Division
DIRECTOR OF DEVELOPMENT

Project 1C522301A05901

August 1964

This publication or any portion thereof may not be reproduced without specific authorization from the Commanding Officer, U. S. Army Biological Laboratories, ATTN: Technical Releases Branch, Technical Information Division, Fort Detrick, Frederick, Maryland. 21701. However, DDC is authorized to reproduce the publication for U. S. Government purposes.

The information in this publication has not been cleared for release to the public.

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this publication directly from DDC.

Foreign announcement and dissemination of this publication by DDC is limited.

ABSTRACT

For the first time, septicemic anthrax established by respiratory exposure has been effectively cured by a treatment combination of antibiotics, antiserum and active immunization during treatment protocol. The treatment protocol is based on the knowledge that death in anthrax is toxemic. Twenty-one of twenty-five monkeys administered these treatment combinations were cured and, by active immunization, were made completely resistant to anthrax upon rechallenging aerogenically with large doses of virulent spores. Of the four monkeys that died, only two died from anthrax and the remaining two from complications. This treatment regime is believed to hold great promise for application to man.

I. INTRODUCTION

Generalized anthrax is a rapidly progressing disease almost invariably fatal. Massive septicemia is characteristic. Specific diagnosis usually depends upon isolating Bacillus anthracis from the blood. The disease is so well advanced before a positive blood culture is obtained that antibiotic treatment is not successful.

It is apparent that other means than the use of antibiotics must be developed to cure septicemic anthrax. This paper presents a treatment for respiratory anthrax of monkeys that has proved effective when administered during the septicemic stage of the disease. Our treatment protocol is based on the knowledge that death in anthrax is caused by toxemia.

Three problems were visualized: (a) prove that anthrax was established and that death would have occurred if the course of the disease had not been changed by treatment deliberately applied; (b) effect a cure after septicemic anthrax was established; and (c) develop procedures to prevent latent or secondary infection occurring after therapy was stopped.

II. MATERIALS AND METHODS

A. EXPERIMENTAL PROCEDURES

Except as noted, 12- to 18-pound mature rhesus monkeys (Macaca mulatta) were used. These monkeys* were challenged via the respiratory route with a calculated inhaled dose of approximately 100,000 anthrax spores of the virulent V1b strain. Commercial Sclavo-type, equine antiserum against the Sterne strain of anthrax was used. Titer of the antiserum was 1:128; approximately 7150 units of toxin were neutralized/ml of antiserum. Blood samples were obtained from the right cardiac ventricle by cannulation through the external jugular vein.

B. EVALUATION OF ANTIBIOTICS

Because other workers have recommended a variety of antibiotics for the treatment of anthrax, it was necessary to determine the drug of choice by in

---

* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.

vivo experiments. Most recommendations in the literature refer to cutaneous anthrax, and there have been few survivors when treatment was begun after septicemia developed.

In our experiments, five drugs were tested using inbred mice (BALB-c strain) challenged intraperitoneally with 100,000 anthrax spores. The drugs and dosages are shown in Table I. When a single dose of antibiotic was given at varying times after challenge, dihydrostreptomycin and oxytetracycline were best (Table II). If a series of 10 antibiotic injections were given, these two antibiotics and penicillin showed up well (Table III). Crystalline penicillin gave a slightly longer survival time than did procaine penicillin. Chloramphenicol was not effective and did not even change the time to death significantly from that of the controls. Chlorotetracycline extended time to death but did not allow survival. We concluded from these data that no one antibiotic was ideal. Therefore, because of the widely observed synergistic action of penicillin-dihydrostreptomycin, we proceeded with this combination of antibiotics, to which we added an initial dosing of crystalline penicillin to get the maximum early effect of antibiotic therapy.

TABLE I. DRUGS AND DOSAGES USED IN TREATING MICE CHALLENGED INTRAPERITONEALLY WITH 100,000 *B. ANTHRACIS* SPORES

Antibiotic	Amount of Antibiotic Used per 20-gm Mouse (in 0.2 ml)	Maintenance Dose
Penicillin crystalline	1000 units	400 units
Penicillin procaine	1000 units	400 units
Dihydrostreptomycin	0.4 mgm	0.2 mgm
Oxytetracycline (Terramycin)	0.4 mgm	0.2 mgm
Chlorotetracycline (Aureomycin)	0.4 mgm	0.2 mgm
Chloramphenicol (Chloromycetin)	0.4 mgm	0.2 mgm
Combination Penicillin procaine and D-h-Strep	1000 units 0.4 mgm	400 units 0.2 mgm

TABLE II. SURVIVAL OF MICE GIVEN A SINGLE DOSE OF ANTIBIOTIC FOLLOWING INTRAPERITONEAL CHALLENGE WITH 100,000 ANTHRAX SPORES (10 MICE PER TREATMENT)

Treatment[a]	Survivors if Treatment Given at Indicated Hours following Challenge, per cent				
	2	4	8	12	16
Penicillin procaine	20	0	50	10	10
Penicillin crystalline	20	10	30	10	10
Dihydrostreptomycin	10	10	30	40	30
Oxytetracycline (Terramycin)	10	20	30	30	50
Chlorotetracycline (Aureomycin)	0	10	0	0	0
Chloramphenicol (Chloromycetin)	0	0	0	0	0
Control 10/10 (MTD[b] - 2 hr)					

a. 10 mice per treatment.
b. Mean time to death.

TABLE III. SURVIVAL OF MICE CHALLENGED IP BY 100,000 ANTHRAX SPORES WHEN TREATED FOR 120 HOURS (10 INJECTIONS AT 12-HOUR INTERVALS)

Antibiotic	Number Dead during 120 Hrs of Treatment	Dying after Treatment was Completed	
		Number	MTD[a] Hours
Penicillin, procaine	18/46	20/34	104
Penicillin, crystalline	13/45	24/32	134
Dihydrostreptomycin	2/43	31/41	232
Oxytetracycline (Terramycin)	1/45	32/44	223
Chlorotetracycline (Aureomycin)	31/50	18/19	93
Chloramphenicol (Chloromycetin)	50/50	-	-
Control	24/24	-	-

a. Mean time to death after treatment stopped.

C. ANTHRAX TOXEMIA

Several workers[1-4] have shown that toxin is present in the blood of animals dying of anthrax. We also showed that toxins build up in the lymph of the anthrax-infected rhesus monkey for many hours before toxin can be demonstrated in the blood. The Fischer strain number 344 rat dies in as short a period as one hour after IV injection of one ml of filtered sterile terminal blood of the rhesus monkey or chimpanzee or of sterile in-vitro-produced toxins.[4-6] It is apparent that if anthrax is to be cured during the later septicemic stages, toxins must be neutralized with specific antiserum, and their toxic effect countered with appropriate chemotherapy.

D. USE OF IMMUNE SERUM PLUS ANTIBIOTICS

The importance of immune serum and the possible interaction with antibiotic were studied in a factorially designed experiment. The variables were antiserum (1 ml/kg) and penicillin (20,000 units/kg), each at (a) zero level, (b) a plus level administered intramuscularly (IM), and (c) a plus level administered intravenously (IV). Four replicate experiments were conducted using 5- to 6-pound rhesus monkeys. Antiserum was administered at 0, 12, and 24 hours. The IV penicillin was given initially IV and the remaining doses IM at 12-hour intervals; penicillin IM was administered at all times IM on a 12-hour schedule. Thus, the penicillin treatments differed only in the route of the initial dose. Data are presented in Table IV.

TABLE IV. SURVIVAL AND TIME TO DEATH OF RHESUS MONKEYS GIVEN INDICATED TREATMENT

	Penicillin					
	None		IV		IM	
Antiserum	Survival[a]	MTD[b]	Survival	MTD	Survival	MTD
None	0/4	73	1/4	112	2/3	201
IV	0/4	55	2/3	172	3/3	S[c]
IM	0/4	44	1/4	202	3/3	S

a. Survived/number tested.
b. Mean time to death, hours.
c. All survived.

Our results showed that the route of administration of penicillin was important, because when penicillin was administered IV, only five of 11 monkeys survived. Eight of nine survived if administration was IM. All monkeys that survived had received penicillin. Antiserum alone was ineffective in preventing death, although a temporary decrease (about 1.5 log) in the number of bacilli/ml of blood was observed. Route of administration of antiserum was unimportant, as 5 of 10 monkeys survived after the IV administration of antiserum, 4 of 11 survived after the IM administration. Of those monkeys receiving both antibiotic and antiserum, 9 of 13 survived.

When the experiment is examined in regard to the monkeys that died, it may be seen that as the efficiency of the treatment increased in terms of promoting survival, so did the time to death increase in those that did not survive; i.e., they lived longer. But where there are survivors, a statistical comparison is difficult. We concluded that penicillin should be administered IM, but that antiserum might be administered by either route. In our later work, we administered both antibiotic and antiserum IM.

E. EFFECT OF HYDROCORTISONE

In recent studies* we have found that the administration of hydrocortisone in pharmacological doses prior to challenge of rats with sterile anthrax toxins extended the time to death significantly. Blood glucose, calcium, and pH levels decreased significantly during the course of disease.* These observations are consistent with the concept that administration of steroids may counteract certain physiological effects of anthrax toxins. In a preliminary experiment, twelve 5- to 6-pound monkeys were challenged IV with 10^7 spores. Six animals were injected IM with 20,000 units of procaine penicillin and six received 20,000 units of penicillin plus hydrocortisone at the rate of 1 ml/pound of weight. This therapy was initiated when the number of organisms/ml of blood was between 10^4 and 10^6 for the animals in each replicate, and was continued at 12-hour intervals. Those receiving penicillin survived for an average time of 34.5 hours after treatment was initiated, and five of the animals given penicillin plus hydrocortisone survived an average of 38 hours. This group had one survivor that is not included in the average figure. At this point we decided that further tests would be required to prove the possible value of steroids in treating septicemic anthrax, and moreover, that the organisms themselves must be under rigid control with antibiotics before steroids could be of value.

F. CONCLUDING CRITICAL EXPERIMENTS

Twenty-five mature rhesus monkeys, in three experiments, were challenged aerogenically with ca 100,000 spores to demonstrate the value of the

---

* Unpublished data.

recommended treatment regime. Blood samples were taken at four-hour intervals until a bacteremia was observed, then at two-hour intervals until the increasing number of bacilli in the blood between successive samplings met the statistical requirements for the doubling rate of a progressing septicemia (a significant increase in number of bacilli per microscopic field). The rate of increase or doubling rate and the allowable variation was generated from data on more than 60 monkeys. The therapeutic regime is outlined in Table V.

TABLE V. THERAPY PROTOCOL USED TO TREAT RHESUS MONKEYS

Penicillin:	Crystalline -	40,000 units/lb at 0 time
	Procaine -	20,000 units/lb at 2, 12, 24 . . . 168 hours, then each 24 hours at one-half this rate.
Dihydrostreptomycin		7.5 mg/lb at 0, 2, 4, 12, 24 . . . 168 hours, IM
Anthrax antiserum		1 ml/lb at 0, 12, and 24 hours, IM
Hydrocortisone (expts. 2 and 3)		10 mg/lb at 0, 4, 12, 24, 36 hours, IV
Also available but not used:		Dextran, epinephrine, oxygen and vitamin B complex

In the first experiment with four monkeys, as indicated in Table VI, a bacteremia was present for at least 16 and for as long as 26 hours before treatment was begun, and at the time treatment was initiated, the blood level varied from 1,160 to 14,650 bacilli/ml of blood. In this experiment hydrocortisone was not used. The number of organisms in the blood had been reduced below a detectable level two hours after treatment was initiated. Three animals survived. One died from an aspiration pneumonia, which resulted from repeated forced feeding. At necropsy of the latter animal, no organism or anthrax toxin could be demonstrated in the blood or spleen.

In the second experiment with 11 monkeys, hydrocortisone was used. Treatment began when the bacilli/ml of blood varied between 100 and 7,500. Eight of 11 monkeys survived. Among the three that died, one died two hours,

TABLE VI. NUMBER OF BACILLI/ML OF BLOOD DURING THE TIME BEFORE TREATMENT OF RHESUS MONKEYS WAS INITIATED

By Direct Microscopic Observation			
Monkey Number	Organisms First Observed at Hours after Challenge	Treatment Started, Hours after First Observation of Organisms	Number of Bacilli per ml of Blood when Treatment Initiated
1	78	18	14,650
2	34	14	3,540
3[a]	48	18	1,160
4	46	26	7,500

a. Died from aspiration pneumonia attributable to forced feeding.

one five days, and one 19 days after treatment began. At necropsy, none of these three animals had detectable anthrax toxin or bacilli in the blood, but the animal that died at two hours, had anthrax bacilli in the spleen. No anthrax bacilli were found but masses of lung mites and gross acarid lesions were observed on necropsy of the monkey that died on Day 19.

In a third experiment in which ten monkeys were challenged, the time of administration of hydrocortisone was tested in a factorial experiment. Since the effect of hydrocortisone was not significant, this variable will not be discussed. The number of bacilli/ml of blood varied between 10 and 4,000. All animals survived.

In summary, as shown in Table VII, 25 animals were challenged and 21 were cured. Of the four dying, two did not die of anthrax.

In reviewing these three experiments, it is well to realize that there was a great deal of variability in the number of organisms per ml of blood at the time of initiating treatments. However, with one exception, all animals had bacilli in the blood for at least eight hours before treatment was given. Bacilli were demonstrated either by the appearance of colonies on agar from a blood sample plated eight or more hours before treatments or by direct observation of blood smears. Six animal's blood was sterile two hours after treatment and the blood of all animals was sterile six hours after treatment.

TABLE VII. SUMMARY OF TREATMENT EXPERIMENTS USING THE RHESUS MONKEY

Experiment Number	Number of Monkeys Challenged	Number of Monkeys Surviving	Cause of Death of Those Likely Not Dying of Anthrax
1	4	3	1 - aspiration pneumonia
2	11	8	1 - lung mite complication
3	10	10	
Total	25	21	2

Reinfection from spores remaining in the lung of surviving animals following challenge with anthrax aerosols has been described by some workers.[7,8] To prevent this from occurring in the surviving monkeys, we began minimum penicillin therapy 21 days after challenge, and at the same time started vaccination with the Belton-Strange antigen. This antigen was administered IM for five successive times at two- or three-day intervals. Just prior to initiation of vaccination, the serum from each monkey was negative when titrated by the Ouchterlony technique but seven days after completing immunization the serum from all animals titrated between 1:64 and 1:128 dilution. Administration of antibiotics was stopped without recurrence of anthrax. Three rechallenges were made during approximately the 2nd, 4th, and 6th month after the original challenge. Rechallenge did not result in establishment of anthrax in these cured and now immunized animals.

III. DISCUSSION AND SUMMARY

The treatment developed and recommended differs from current usage in recognizing: (a) the extraordinarily rapid progress of this disease once it becomes septicemic; (b) that death from anthrax is due to a toxemia, (c) that effective treatment must include neutralization of toxins and counteraction of unfavorable physiological effects; and (d) that bacilli as generators of toxins must not only be eliminated but eliminated rapidly.

Immediate treatment is essential. When detection is based on direct microscopic observation of bacilli, the septicemia progresses in about 12 hours from the minimum observable level of about 10,000 bacilli/ml of blood to the average terminal number of 1×10^7 bacilli/ml of blood. This leaves only a few hours to initiate therapy that can be effective.

Once the disease is recognized and the decision is made to initiate treatment, a massive dose of crystalline penicillin should be given immediately. The level of antibiotic should be maintained by frequent dosing, and by shifting to long-acting penicillins after the septicemia is brought under control. We believe that penicillin and dihydrostreptomycin are synergistic in action and that both antibiotics should be used at the same time. The tetracycline-type antibiotics gave the next most satisfactory results. Chloramphenicol did not cure generalized anthrax in monkeys.

The classical antidote or neutralizer for toxin is an antitoxin, and specific immune antiserum is the only neutralizer of anthrax toxin known. In fact, the cures effected by antiserum alone in the 1920's and 1930's appear to be greater than those following use of modern antibiotics in the 1940's and 1950's. In our work the amount of antiserum used was deliberately kept minimal, although undoubtedly use of greater quantities would be beneficial. It is our premise that antiserum should be present at the time the bacilli are lysed by antibiotics so that the liberated toxins may not become fixed to body cells. Steroids seem to have had a favorable effect if administered after the bacteremia was brought under control.

After control of the septicemia and toxemia, relapse was prevented by a regime of penicillin and vaccination. With this combination of treatments, we now for the first time have been able to cure monkeys of respiratory anthrax that has progressed into the septicemic stage. We believe this holds great promise for application to man.

THIS
PAGE
IS
MISSING
IN
ORIGINAL
DOCUMENT

LITERATURE CITED

1. Smith, H.; Keppie, J.; and Stanley, J.T. "The chemical basis of the virulence of Bacillus anthracis: V. The specific toxin produced by Bacillus anthracis in vivo," Brit. J. Exptl. Pathol. 36:460-472, 1955.

2. Thorne, C.B.; Molner, D.M.; and Strange, R.E. "Production of toxin in vitro by Bacillus anthracis and its separation into two components," J. Bacteriol. 79:450-455, 1960.

3. Klein, F.; Hodges, D.R.; Mahlandt, B.G.; Jones, W.I., Jr.; Haines, B.W.; and Lincoln, R.E. "Anthrax toxin: Causative agent in the death of rhesus monkeys," Science, 138:1331-1333, 1962.

4. Beall, F.A.; Taylor, M.J.; and Thorne, G.B. "Rapid lethal effect in rats of a third component found upon fractionating the toxin of Bacillus anthracis," J. Bacteriol. 83:144-152, 1962.

5. Klein, F.; Haines, B.W.; Mahlandt, B.G.; DeArmon, I.A., Jr.; and Lincoln, R.E. "Dual nature of resistance mechanisms as revealed by studies of anthrax septicemia," J. Bacteriol. 85:1032-1038, 1963.

6. Echert, N.J., and Bonventre, P.F. "In vivo effects of Bacillus anthracis culture filtrates," J. Infect. Diseases 112:226-232, 1963.

7. Henderson, D.W.; Peacock, S.; and Belton, F.C. "Observations on the prophylaxis of experimental pulmonary anthrax in the monkey," J. Hyg. 54:28-36, 1956.

8. Berdjis, C.G.; Gleiser, C.A.; Hartman, H.A.; Knehne, R.W.; and Gochenour, W.S. "Pathogenesis of respiratory anthrax in Macaca mulatta," Brit. J. Exptl. Pathol. 43:515-524, 1962.